植物研究雑誌
J. Jpn. Bot.
67: 63–71 (1992)

Review

Renewed Studies on Some Lichen Metabolites and Their Development

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過去の地衣成分研究の再検討とその新しい展開

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(Received on November 9, 1991)

In response to the criticisms by the modern concepts, renewed investigations have been performed on some lichen substances studied earlier by the author. The original structures of psoromic acid and didymic acid were reconfirmed by the present knowledge and the methods currently used. Retigeranic acid was separated by HPLC into two stereoisomers, A and B, whose structures were established by X-ray crystallography. As a new development of earlier study, an antiviral agent against human immunodeficiency virus (HIV-1), GE-3-S, was induced from a host-mediated anti-tumour active lichen polysaccharide, GE-3.

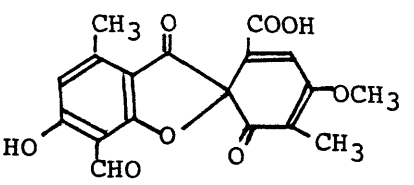
Since Professor Asahina and I published "Chemistry of Lichen Substances" in 1954, in which about 75 lichen substances with established chemical structures were recorded (Asahina and Shibata 1954), several review articles have been published to follow the progress of studies on lichen metabolites (Culberson 1969, Huneck 1973, Elix, Whitton and Sargent 1984). The latest review of Elix et al. includes about 346 lichen metabolites. Meanwhile, I proposed a biogenetical systematization of lichen substances to elucidate their structural correlation on the basis of biosynthetical schemes classified into acetate-malonate, shikimate, mevalonate and amino acids pathways (Shibata 1965). In earlier investigations of lichen substances, classical chemical methods were mostly employed, and modern spectroscopic analysis and chromatographical techniques have been introduced into this field only after the World War II. My coworkers and I applied NMR spectrometric analysis at first in 1960s to elucidate structural correlation of usnic acid, iso-usnic acid and their dihydroderivatives (Shibata et al. 1962, Shibata and Taguchi 1967). Thereafter, the general application of IR, NMR and Mass spectrometry to the structural elucidation of lichen metabolites was reviewed (Shibata 1978). Recently, our earlier works of some lichen metabolites have been

criticized sometimes in view of the modern concepts. Then appropriate response should be given reexamining the earlier results by the present knowledge and the methods currently employed.

In this article I would like to give some examples of renewed studies on the lichen substances on which I had once been engaged.

Psoromic acid – In 1939 Prof. Asahina and I reported revised structure of psoromic acid, a depsidone isolated from *Alectoria sulcata,* on the basis of the formation of a xanthone derivative from a diphenyl ether carboxylic acid derivative derived from hypopsoromic acid (Asahina and Shibata 1939). Psoromic acid had been represented earlier as a formula having carboxyl at the ortho-position of depside linkage by reason of the formation of phthalic anhydride on pyrolysis. The revised formula explained this by the classical salol rearrangement.

Later, Dean proposed an alternative structure of grisadienedione type for psoromic acid to



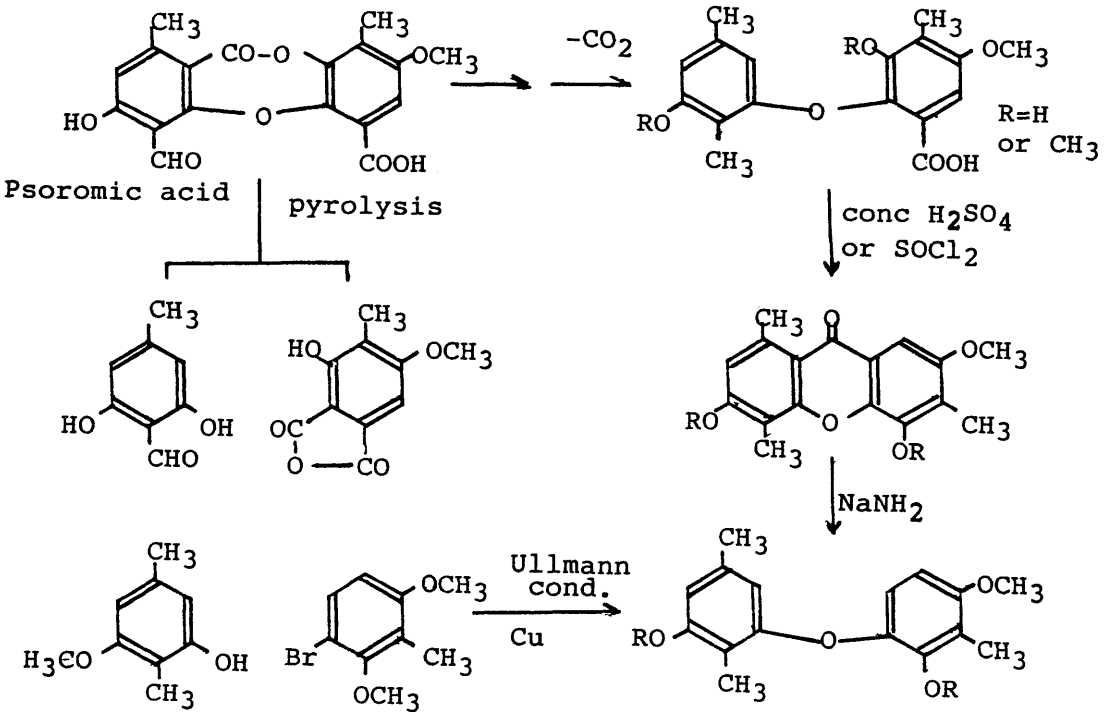
explain the formation of the phthalic anhydride derivative on pyrolysis (Dean 1963).

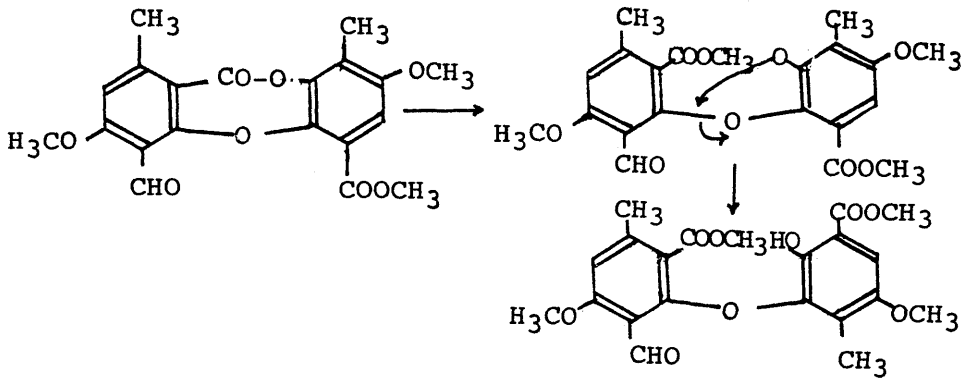
But in 1976 Huneck and Sargent reconfirmed our structure of psoromic acid by the spectroscopic data and degradation reactions in which a Smiles rearrangement was involved by the methanolysis of methyl O-methylpsoromate.

They assumed the intermediate formation of grisan on the pyrolysis of psoromic acid.

Sala and Sargent (1979) synthesized psoromic acid by a few steps of selective functionalization reactions to reconfirm the structure synthetically.

Didymic acid – The structure of didymic acid isolated from *Cladonia pseudodidyma, Cl. floerkeana,* var. *suboceanica, Cl. bacillaris* var.





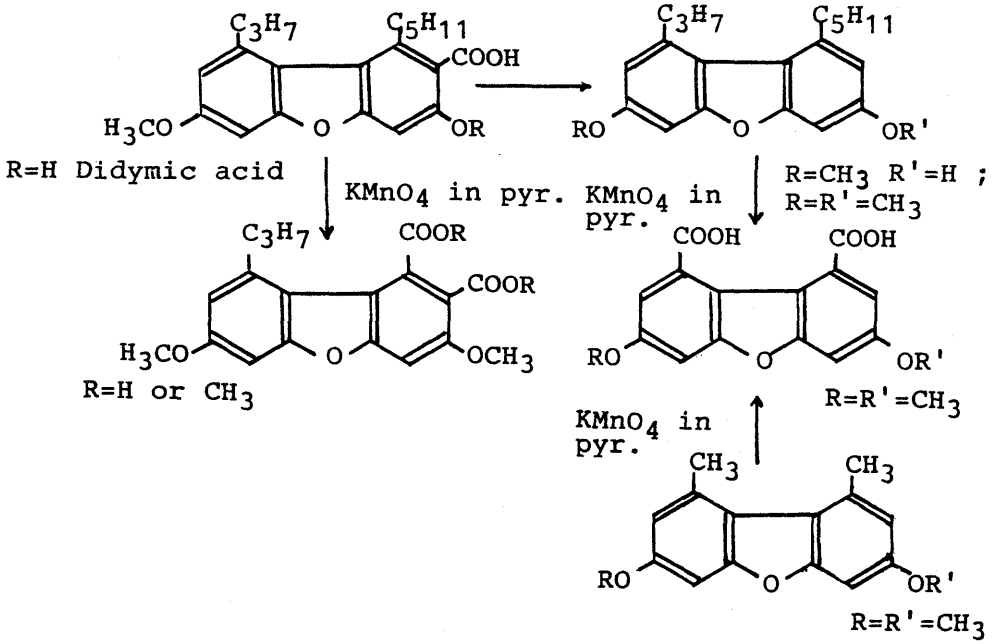
pacifica and *Cl. incrassata* was put forward in 1944 by the following chemical reactions (Shibata 1944):

The carbonskeleton and the disposition of alkyl(n-C_3H_7 and n-C_5H_{11}) groups in didymic acid molecule were determined by the derivation from synthesized 1,9-dimethyldibenzofuran. The special correlation between COOH and OH groups was proved by the blue coloration with $FeCl_3$, and the vicinal disposition of n-C_5H_{11}, COOH and OH was deduced from a characteristic behaviour of didymic acid forming three layers at the extraction from the ethereal solution with aq. $NaHCO_3$.

On controlled oxidation of didymic acid methyl ether with $KMnO_4$ in pyridine, the formation of an ortho dicarboxylic acid derivative was assumed to support the positions of C_5H_{11} and COOH groups.

In 1983 Sargent attempted to synthesize didymic acid and proposed an alternative formula having n-C_3H_7 in 1-position and n-C_5H_{11} in 9-position by reason of the different melting point of his synthesized product with the data which I reported earlier for natural didymic acid.

Because of shortage of the material, I could not



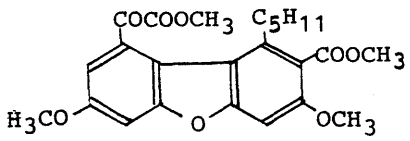
effectively response to his demand for direct comparison of natural and synthetic didymic acid. IR, ^{1}H NMR and ^{13}C NMR spectral analysis, which had not been used for didymic acid in earlier days, are not effective for the determination of alternative disposition of two alkyl groups on the dibenzofuran ring. Mass spectral analysis, which seemed to give a conclusive evidence, has not been reliable because of difficulty in electronic cleavage of the dibenzofuran ring.

Consequently, I used X-ray crystallography for methyl ester of the controlled oxidation product, which was assumed to have an ortho dicarboxylic acid system. The result given by the collaboration of Prof. Y. Iitaka showed that the compound should be formulated as below revising the structure proposed earlier (Shibata and Iitaka 1984).

In spite of the structural correction of the oxidation product, the original didymic acid structure has been proved to be correct including the disposition of n-C_5H_{11} group. Interestingly enough, the calculated C, H values in the elementary analysis of methyl ester of the controlled oxidation product (Obs. C 65.51, H 6.08) are adopted within the error to either the new, $C_{24}H_{26}O_8$, or the earlier molecular formula, $C_{21}H_{22}O_7$ (Anal calcd. C 65.28, H 5.70).

Retigeranic Acid – My coworkers and I proposed in 1972 (Kaneda, Takahashi, Iitaka and Shibata 1972) a novel sesterterpene structure for retigeranic acid isolated from *Lobaria retigera* and *L. isidiosa* var. *subisidiosa*. This structure was confirmed by X-ray crystallographic analysis of its *p*-bromoanilide (Kaneda, Iitaka and Shibata 1974). Recently, Corey and his collaborators (Corey, Cesai and Engler 1985) attempted a total synthesis of racemic form of this compound, and Paquette and his collaborators (Wright, Drtina, Roberts and Paquette 1988) also tried to perform an enantiospecific total synthesis of the same compound.

In response to Prof. Corey's request I sent my sample of retigeranic acid, and soon he found that methyl ester of my sample showed two peaks on the HPLC, one major (r.t. 30 min) and one minor (r.t. 31.2 min), and the minor one was identical



Methyl ester of a controlled oxidation product of methyl O-methyldidymate

X-ray crystallographical data:
Mol formula: $C_{24}H_{26}O_8$;
mol. wt.: 442;
Anal calcd. C 65.15, H 5.92
Monoclinic; space group Pz_1/n;
$z=4$, $D_{calcd.}=1.299$ gcm^{-3},
$a=19.522(10)$, $b=8.271(4)$,
$c=14.153(7)$Å; $\beta=97.99(5)$; $V=2263$Å;
ν for CuK$\alpha=7.74$ cm^{-1}

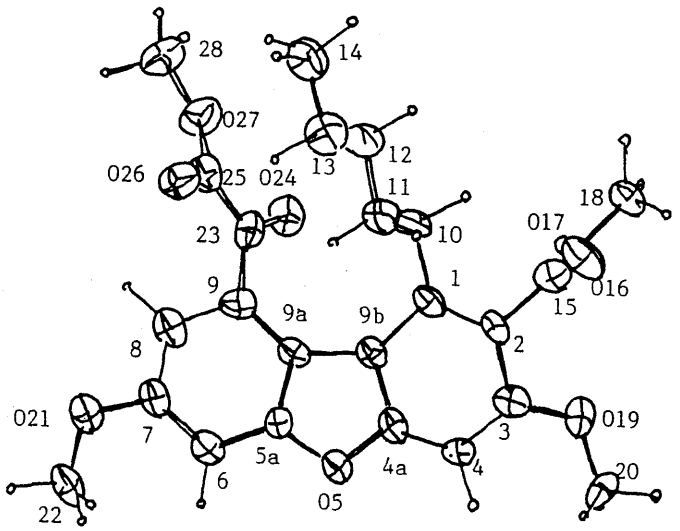


Fig. 1. A perspective drawing of the molecule of the methyl ester of a controlled oxidation product yielded from methyl O-methyldidymate.

with methyl ester of his synthesized product.

I examined my original sample of retigeranic acid which gave a single spot on TLC, but it was separated into two peaks on HPLC over an ODS column. The two components, *A* and *B*, gave superimposable UV curves and the same Mass number.

I sent my original sample of *p*-bromoanilide of retigeranic acid, which was used for X-ray analysis, to Prof. Corey to be identified with that of his synthetic retigeranic acid *p*-bromoanilide. It was HPL-chromatographically identical with *p*-bromoanilide of his synthetic compound. It has now become clear that we had determined X-ray crystallographical structure of *p*-bromoanilide of the minor component (*A*) of natural retigeranic acid which might readily crystallize out from the mixture solution.

Then X-ray crystallographical analysis of two components (*A* and *B*) of natural retigeranic acid has been performed. The major component (*B*), which is now designated retigeranic acid B, m.p. 220–222°, $[\alpha]_D$ −30.4° (EtOH), has been subjected to X-ray crystallographic analysis to have β-isopropyl, *R* at C(18), and the minor component (*A*), retigeranic acid A, m.p. 188–190°,

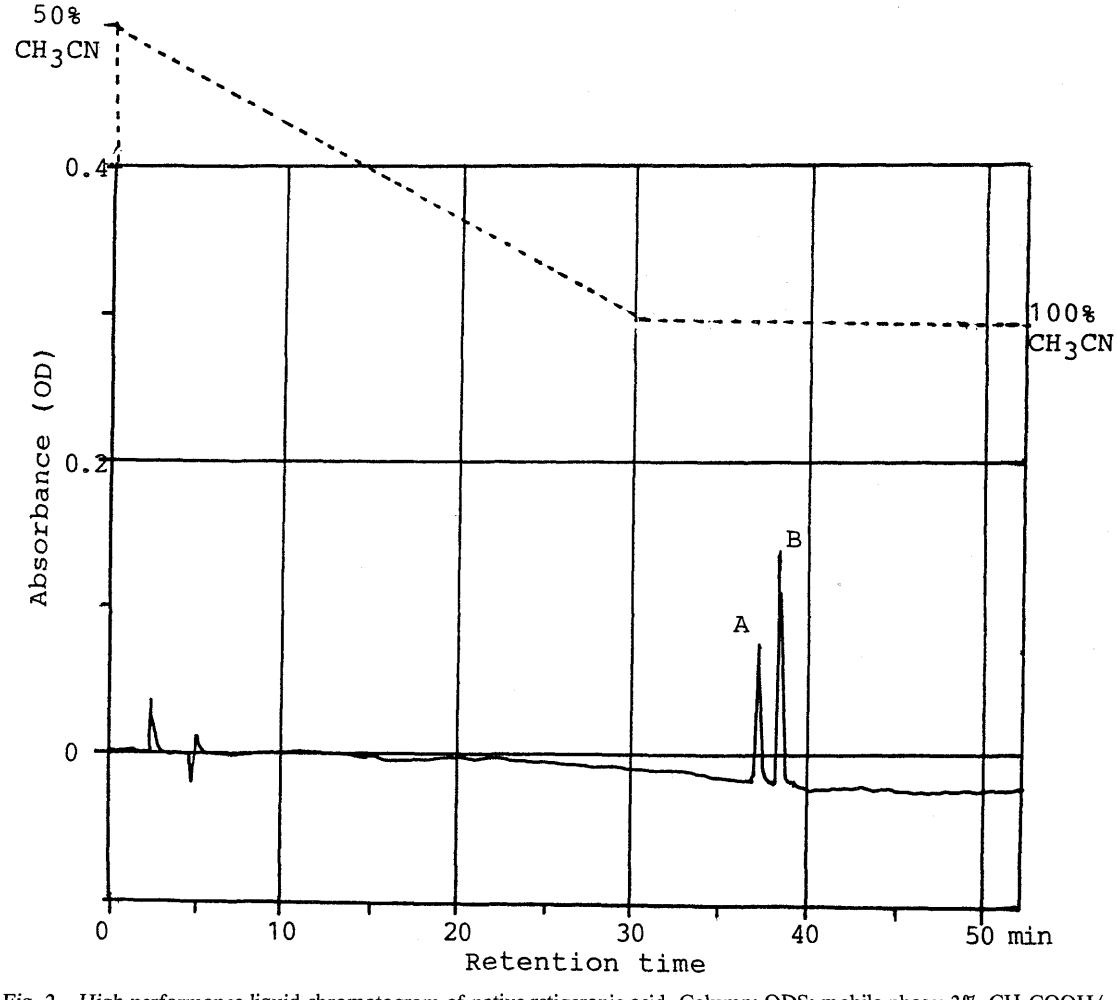
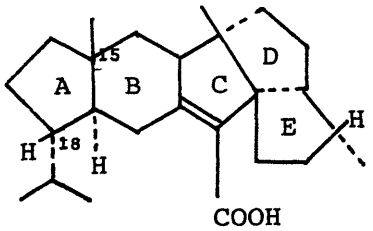
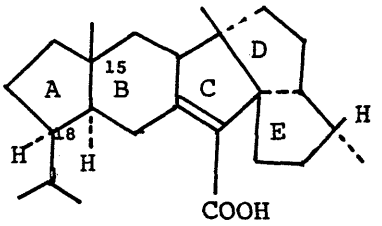


Fig. 2. High performance liquid chromatogram of native retigeranic acid. Column: ODS; mobile phase: 3% CH_3COOH/CH_3CN. UV: 253 nm.



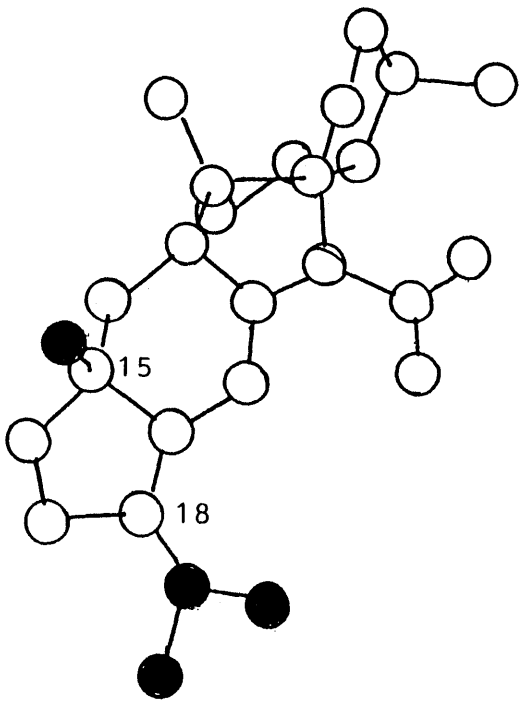
Retigeranic acid A
S at C_{18}
(minor)



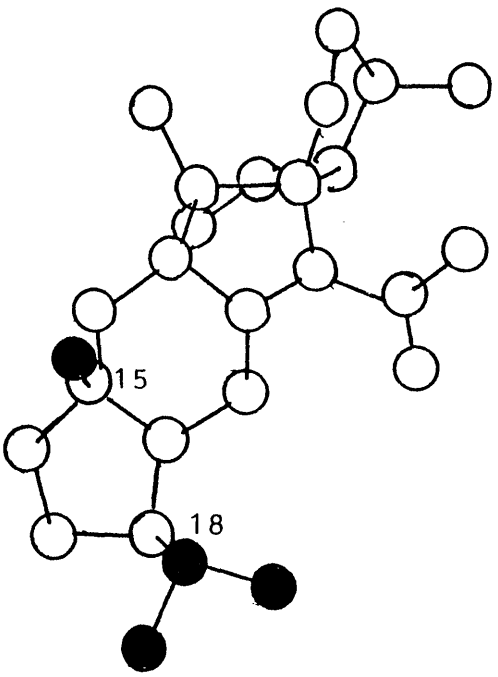
Retigeranic acid B
R at C_{18}
(major)

$[\alpha]_D$ −86.5° (EtOH), to have α-isopropyl against methyl group at C(15), *S* at C(18).

According to Corey's synthetic process starting with Δ^5-3-α-isopropyl-9-methyl hydrinden-6-one, it is obvious that he synthesized retigeranic acid A, the minor partner, whose stereochemistry was previously illustrated in our earlier paper as retigeranic acid deduced from X-ray analysis of its *p*-bromoanilide. Paquette and his coworkers (Wright, Drina, Roberts and Paquett 1988) synthesized enantiometrically pure retigeranic acid A and its stereo-isomers at A/B and B/C ring junctures.



Retigeranic acid A
m.p. 188–190°
$[\alpha]_D$ − 86.5° (EtOH)



Retigeranic acid B
m.p. 220–222°
$[\alpha]_D$ − 30.4° (EtOH)

Fig. 3. The stereochemistry of retigeranic acid A and B.

The biological activity of the lichen polysaccharide, GE-3 and its sulphate (GE-3-S)

It is well known that lichens contain polysaccharides, homo- and heteroglycans, in a fairly high percentage besides lichen metabolites of smaller molecular size.

Some years ago, my coworkers and I found that some β-glucans in lichens show host-mediated anti-tumour activities against implanted Sarcoma 180 and Ehrlich's carcinoma of mice (Shibata et al. 1968, Fukuoka et al. 1968). Especially GE-3, partially acetylated pustulan, a β(1-6)-glucan, isolated from *Umbilicaria esculenta* (= *Gyrophora esculenta*)), showed a constant and noticeable anti-tumour activity by i.p. administration to ICR mice (Shibata, Nishikawa, Takeda and Tanaka 1968, Nishikawa, Takeda, Shibata and Fukuoka 1969). It has been shown recently that the anti-tumour activity of the polysaccharides would be caused by the biological response modifiers induced by an inflammatory change in liver occurring immediately after i.p. injection of the reagent.

Macroscopical increase of the liver weight of mice was observed by i.p. administration of GE-3, 50 mg/kg/d, and temporary increase of leucocytes followed by excretion of α_1-acid glycoprotein in the serum on day 6–9 was shown. By electrofocussing the mouse α_1-acid glycoprotein was separated into α_1-AG-1 (IEP 3.2) and α_1-AG-2 (IEP. 3.4), and α_1-AG-1 inhibited *in vitro* the growth of L 1210 cells at the concentration of 200 μg/ml. Therefore, α_1-AG-1 may play a significant role in the host-mediated anti-tumour activity of GE-3. The similar biological responses were shown by i.p. administration of lichenan, a β(1-3)(1-4)glucan, and pachyman, a β(1-3)glucan, to mice (Watanabe, Iwai, Shibata, Takahashi, Narui and Tashiro 1986).

Recently, chemical inhibitors against human immunodeficiency virus (HIV), which is now considered to be the aetiological agent of acquired immune deficiency syndrome (AIDS), has extensively been investigated. Several anti-HIV compounds, ribavirin, phosphonoformic acid, recombinant interferon-α, 3'-azido-2',3'-dideoxythymidine (AZT) and glycyrrhizin (Ito, Nakashima, Baba, Pauwels, Declereq, Shigeta and Yamamoto 1987), have already been tested against AIDS. It has also been noted that heparin and sulphate of dextran, α(1-6)glucan, are effective to suppress the infection and replication of HIV (Ito, Baba, Sato and Pauwels 1987). It prompted us to study the anti-HIV activity of lichen polysaccharides and their sulphates. Recent experimental results showed that sulphated GE-3(GE-3-S) is the most promising agent for HIV suppression (Hirabayashi, Iwata, Ito, Shigeta, Narui, Mori and Shibata 1989).

GE-3-S (S content 13.8% $[\alpha]_D$ −25.0°, mol. wt. 200,000) was prepared by sulphonation of GE-3 using chlorosulphonic acid.

For testing the anti-HIV activity of GE-3-S, the HTLV-I carrying cell line, MT-4, and the human leukemic T-cell line, Molt-4 (clone 8) were used. The cells were cultured in RPMZ-1640 medium supplemented with 10% fetal calf serum (FCS), 100 IU penicillin G and 100 μg/ml streptomycin. The HIV was obtained from the culture supernatant of the Molt-4/HTLV-III cell line.

Anti-viral activity of GE-3-S was determined by trypanblue exclusion using a haematocytometer. Molt-4 (clone 8) cells were infected with HIV at MOI of 0.002 and incubated for 1 hr at 37°C. After washing, the infected cells were suspended in a culture medium. The number of cells was adjusted to 1×10^5 cells/ml, and the cells were brought into each well (90 wells microtiter) containing various concentrations of GE-3-S, other lichen polysaccharides and their sulphates. After incubation at 37°C for 4 days in a CO_2-incubator, half of the culture medium was exchanged with the fresh

medium. After incubation for another 2 days at 37°C, the number of viable cells was counted microscopically by trypanblue exclusion method.

Only 3×10^5 cells/ml of Molt-4 (clone 8) cells can survive on day 3 after HIV-infection as compared with 10×10^5 cells of the uninfected control. On the other hand, in the presence of 35 μg/ml of GE-3-S the host cells were completely protected against cytopathic effect of HIV. GE-3-S at this concentration gave no cytotoxic effect on the host cells. The immunofluorescene (IF) method showed that 52.4% of Molt-4 (clone 8) cells expressed viral antigens on day 3 of HIV-infection, whereas in the presence of 31 μg/ml of GE-3-S the viral antigen expression was almost completely suppressed.

GE-3-S at the same concentration completely inhibited the formation of giant cells of Molt-4 (clone 8) induced by the infection of HIV. In comparison with other lichen polysaccharides and their sulphate so far examined, GE-3-S is the only one that exhibited anti-HIV activity.

The presence of sulphate group in the molecule of GE-3-S is essential for the anti-HIV activity, since GE-3 is not effective. It has been known that dextran sulphate and heparin are also suppressive against HIV infection. All these polysaccharide sulphates expressed no inhibitory effect on the cell-free HIV reverse transcriptase activity. Therefore, these polysaccharide sulphates would interfere the adsorption of HIV particles to the surface of T-4 cells, probably at the site of the viral envelope gp 120.

I wish to thank Prof. Yoichi Iitaka for his cooperation for X-ray crystallographical analysis. Thanks are also due to all the collaborators, whose names are cited in the references, for persuing the works elucidated in this article.

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要 旨

著者らが以前に発表した地衣 *Alectoria sulcata* 等の成分の psoromic acid, *Cladonia pseudodidyma* 等の成分 didymic acid の構造については近年になって異論が提出されたが, 何れも新しいスペクトル分析あるいはX線結晶解析の手法により従来の構造式の正しいことが証明された. また *Lobaria retigera* 群の特異な sesterterpene, retigeranic acid, は高速液体クロマトグラフによってA, B2種の異性体に分離されその各々が isopropyl 基を含む C-18位に於ける *S* および *R* の立体異性体であることがX線結晶解析で立証された. また宿主仲介抗腫瘍性を示した *Umbillicaria esculenta* の地衣多糖体 GE-3 を硫酸化した GE-3-S が in vitro で抗 HIV 活性を示すことが認められた.